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14. ABSTRACT

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We successfully set up a humidity control system which can control the humidity of the CD sample compartment. Humid air is harmful to the optical components of the CD spectrometer. Therefore instead of building a humidity control system inside the CD spectrometer, we built an air tight chamber in the sample compartment, a humid air balance box with humidity control device outside of the CD spectrometer. We connected these two parts using light resistant tubes.

We successfully applied this newly installed CD system to study various surface immobilized peptides and proteins. We successfully studied secondary structures of surface immobilized peptides/proteins with different surface immobilization methods, on different immobilization substrates, at different environmental conditions, and with or without co-immobilized hydromimetic functionalities.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

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(c) Presentations

Number of Presentations: 0.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

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Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

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TOTAL:

Number of Manuscripts:

Books

Received Book

TOTAL:

Received	Book Chapter

TOTAL:

Patents Submitted

Patents Awarded

Awards

Graduate Students

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Total Number:

Names of Post Doctorates

NAME	PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Names of Faculty Supported

NAME	PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Names of Under Graduate students supported

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FTE Equivalent:
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Names of Personnel receiving masters degrees

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Total Number:

Names of personnel receiving PHDs

NAME

Total Number:

Names of other research staff

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Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

See Attachment.

Technology Transfer

Dr. Lauren Soblosky, a postdoc at Army Natick Soldier Center, came to our lab and received training on using this CD spectrometer. She is studying surface immobilized antimicrobial peptides.

Grant Information

Award Number	W911NF-14-1-0413
Title of Research	Acquisition of a Circular Dichroism (CD) Spectrometer to Study Biological Molecules at Interfaces
Principal Investigator	Zhan Chen
Organization	University of Michigan

Technical Section

Technical Objectives

The objectives of this research include:

(1) We will purchase and set up a state-of-the-art CD spectrometer that has a superb sensitivity in detecting peptide/protein secondary structures. This CD spectrometer is sensitive enough to measure secondary structure of a monolayer of peptides or proteins immobilized on a surface, which will be used to investigate immobilized peptides with different secondary structures as well as immobilized proteins.

(2) We will build a humidity control system to modulate the humidity level of the CD sample compartment. This humidity control system will generate humid air with controlled humidity level and transport the humid air to the CD sample compartment. The effect of humidity on the immobilized peptide and protein conformation will be studied.

(3) We will apply this CD spectrometer to investigate I) the effect of immobilization via different peptide termini on the immobilized peptide conformation, II) the effect of the environmental humidity change on the immobilized peptide and protein conformation, and III) the different immobilized protein structure caused by different immobilization methods: chemical immobilization vs. physical adsorption.

The overall research goal of this proposal is to design and install a CD spectrometer equipped with humidity controllable chamber to investigate the structure and conformation of surface immobilized peptides/proteins. The structure/conformation information of the immobilized peptides/proteins will be coupled with orientational information characterized by sum frequency generation vibrational spectroscopy (SFG) and attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR), which will provide complete structural and oriantational information to elucidate the structure-function relationships of surface immobilized peptides/proteins.

Technical Approach

Currently, the work at the PI's group has focused on understanding how the structure-activity relationship of peptides/proteins immobilized on abiotic surfaces depends upon a) the chemical and physical nature of the abiotic surface; b) the physicochemical properties of the protein or peptide; and c) the nature and effect of the surrounding medium – water, air or other hydromimetic molecules. The combination of SFG and ATR-FTIR has been used to investigate the molecular conformation and orientation of the surface immobilized peptides/proteins. SFG signal can only be generated from a medium without inversion symmetry, thus, a medium which possesses inversion symmetry could not generate SFG signal. Therefore an absence of SFG signal from a particular secondary structure may not mean that there is no such a secondary

structure at the interface. No SFG signal detected may just mean that such secondary structures exhibit inversion symmetry at the interface. Therefore SFG results alone may not provide detailed structural information for each secondary structure at an interface. Using a linear optical ATR-FTIR spectroscopy, peptide/protein structural information at interface may be determined. The amide I vibrational peak centers of different peptide/protein secondary structures are usually different, which can be used to identify different secondary structures. However, such peaks can be quite broad, which may not reliably distinguish different secondary structures.

Circular dichroism (CD) spectroscopy is a powerful technique which detects the difference in absorption of chiral molecules using left and right circularly polarized light beams, which is very sensitive to the secondary structures of peptides/proteins. Extensive research has been reported in the literature to quantitatively determine peptide/protein secondary structures using CD spectroscopy.

Unlike the conventional CD spectrometers that only detect peptide/protein structures in bulk solution, the CD system proposed to set up in our laboratory will be used to study secondary structures of surface immobilized peptides/proteins. Due to the limited amount of molecules immobilized on a single surface, it is difficult to generate sufficient high signal to noise ratio for quantitative analysis. Thus, it is necessary to place multiple samples in the light path in the CD spectrometer to generate strong signals. Here, an adjustable sample holder was included in the implementation of the CD spectrometer. Multiple samples (up to 30 sample substrates) in the light path can generate much stronger signal than that generated from one single substrate for the quantitative analysis.

In addition to the implementation of CD spectrometer in this research, a humidity control system will also be set up for the CD system. Humidity is an important factor that affects peptide/protein structure and orientation at abiotic surfaces. The humidity control system can be used to provide humid air with varied humidity levels in the sample compartment for the CD spectrometer to detect the slight structural changes of surface immobilized peptides/proteins caused by the change of environmental humidity.

Progress Statement Summary

Throughout the year, we have made substantial progress in the design, implementation, and use of our purchased CD system and the humidity control device. Currently this CD spectrometer can be used to study peptide/proteins in solution, on surface, and at different humidity levels.

In order to demonstrate that our CD spectrometer is sensitive enough to detect peptide/protein secondary structure after surface immobilization, we first studied how many surfaces stacked on the light path can generate enough signal for peptide/protein secondary structure analysis and how the quality of the quartz substrate affects the signal to noise ratio of the collected CD spectra. We found that at least 15 pieces of the “low purity” quartz slides (purity is around ~95% - very cheap) or at least 3 pieces of the high purity quartz slides (purity is above 99.9% - much more expensive) are required to immobilize peptides/proteins to produce strong enough signal (190 nm-240 nm).

Also, we successfully set up a humidity control system which can control the humidity of the CD sample compartment. Humid air is harmful to the optical components of the CD spectrometer. Therefore instead of building a humidity control system inside the CD spectrometer, we built an air tight chamber in the sample compartment, a humid air balance box with humidity control

device outside of the CD spectrometer. We connected these two parts using light resistant tubes.

We successfully applied this newly installed CD system to study various surface immobilized peptides and proteins. We successfully studied secondary structures of surface immobilized peptides/proteins with different surface immobilization methods, on different immobilization substrates, at different environmental conditions, and with or without co-immobilized hydromimetic functionalities.

Progress

1. Successfully Set Up a CD Spectrometer with Adjustable Sample Holder and Humidity Controllable Sample Compartment.

We purchased a JASCO-J1500 CD spectrometer containing an adjustable sample holder, which is good for the measurement of both solution samples and surface samples. We designed an air tight sample compartment and humidity control box to control the humidity level of the sample compartment. The picture of this new system is shown in Figure 1. As we stated in the submitted proposal, this new CD spectrometer has several advantages compared to the other commercial CD systems:

(1) We are developing new bioactive devices which can preserve the native structures of surface immobilized peptides/proteins in air at different humidity levels. This CD system which we have built is equipped with a digital humidity controller. This system is ideal to study secondary structures of surface immobilized peptides/proteins under different environmental conditions.

(2) The two most important specifications of a CD instrument are excellent sensitivity and stray light control. The J-1500 has the highest sensitivity and lowest stray light specifications: RMS noise: Less than 0.004 mdeg at 185 nm, 0.007 mdeg at 200 nm, 0.007 mdeg at 500 nm (spectral bandwidth 1 nm, DIT 8 sec) while maintaining stray light less than 0.0003% at 200 nm. This is required for accurate determination of protein secondary structure especially when the CD signal is weak.

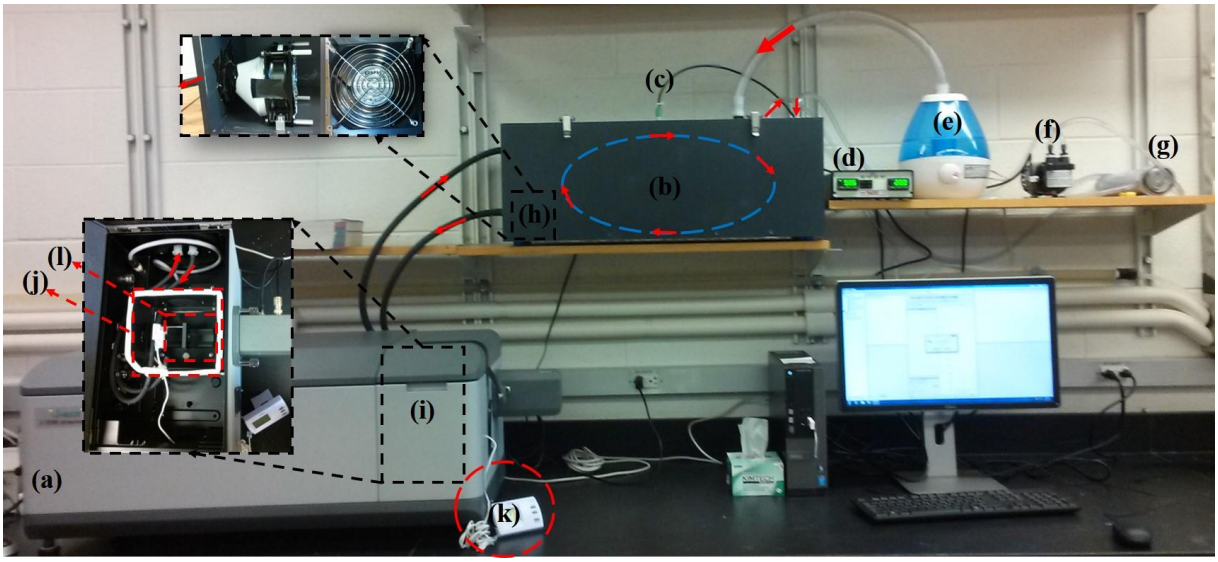

Figure 1. The new CD spectrometer which has been set up in the PI's group. The components of the CD system include (a) main CD spectrometer, (b) humidity control box to generate humid air, (c) humidity sensor of the humidity control box, (d) humidity controller, (e) humidifier, (f) pump, (g) drier, (h) fan for air circulation and transportation, (i) sample compartment, (j) air tight space in sample compartment, (k) hygrometer to monitor humidity level in air tight sample compartment, and (l) adjustable sample holder for samples in solution and on surfaces.

2. Investigate the Secondary Structure of Peptides/Proteins Immobilized on Surfaces

2.1 Study of Substrates Used for Peptide/Protein Immobilization

In order to study the secondary structures of surface immobilized peptides/proteins, quartz slides were used as substrates because of their transparency nature at far UV region (180 nm-240 nm, the region for peptide/protein secondary structure study using CD).

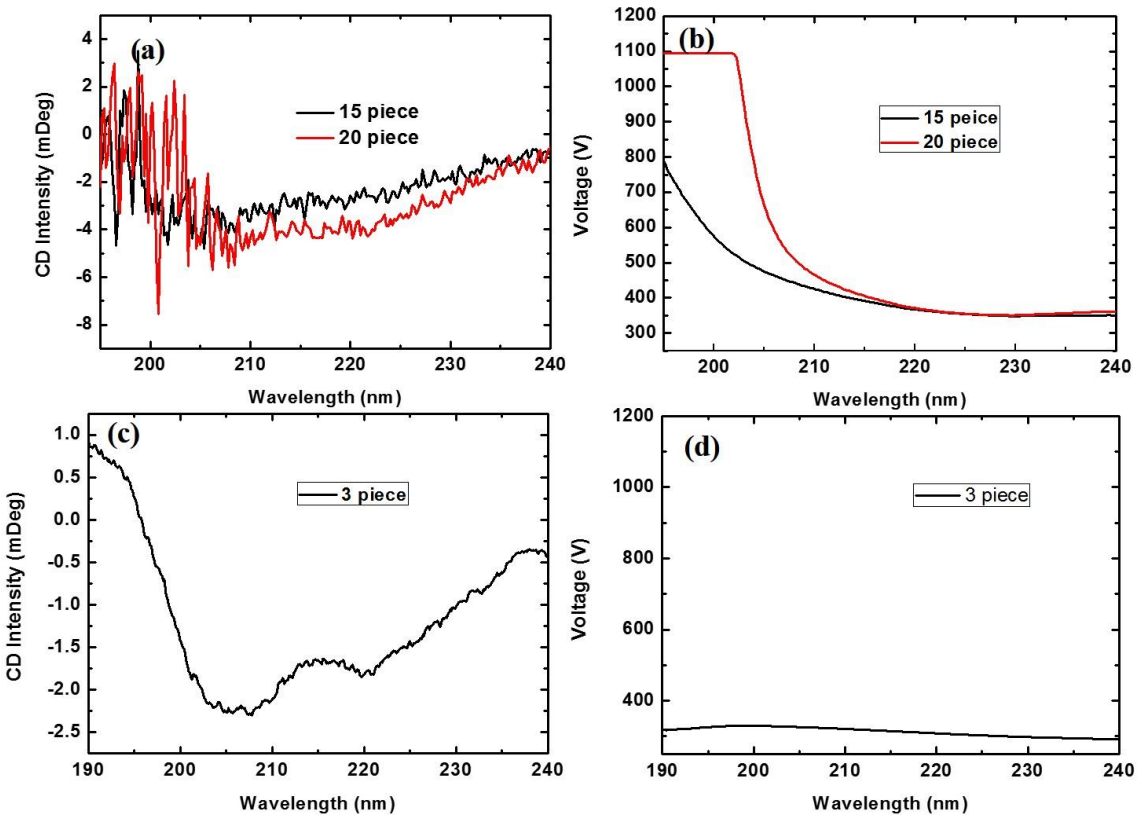

Figure 2. CD spectra of Cecropin P1 immobilized on (a) low quality quartz, and (c) high precision quartz, and the voltage of CD detector for CD spectra collection on (b) low quality quartz, and (d) high precision quartz.

Figure 2 shows our results of comparing CD spectra using low quality quartz slides (contains around 95% SiO_2 – cheap, about \$2/piece) and high precision quartz slides (contains around 99.9% SiO_2 – expensive, about \$50/piece). CP1c was used as a model peptide for surface immobilization. For low quality quartz slides, we need to stack at least 15 pieces of substrates with immobilized CP1c in the light path to generate secondary structure signal. However, the signal to noise ratio is low in the whole scan range. The more quartz slides were used, the stronger noise was generated < 210 nm (Figure 2a). Figure 2b shows the voltage of CD detector for detecting CP1c immobilized on low quality quartz slides. A voltage higher than 800V

suggests the detected CD signal is unreliable. Results here show that the low quality quartz slides cannot be used to study random coil structure (representative peak is at ~197 nm) of the immobilized peptides/proteins. For high quality quartz slides, a minimum of 3 slides can generate strong enough secondary structure CD signal from immobilized peptides/proteins for analysis. Results in Figure 2c and d show that spectrum collected for peptide on high precision quartz has stronger signal and much higher signal to noise ratio. In our current studies, we stack 3 to 5 high precision quartz slides with immobilized peptides/proteins to collect CD spectra for secondary structure analysis.

2.2 Study Surface Immobilized Peptides/Proteins.

2.2.1. Immobilization of Peptide on Pure and Mixed SAMs with Maleimide Groups

CD spectroscopy is very sensitive to the secondary structures of peptides and proteins, which can be used to distinguish random coil, α-helical, and β-sheet secondary structures. Figure 3 shows the CD spectra collected from immobilized C-terminus cysteine modified Cecropin P1 (CP1c, sequence is shown in Table 1) on a maleimide terminated SAM (Mal SAM) and a maleimide-hydroxyl (Mal-OH) mixed SAM exposed to PBS to study their secondary structures. It was shown that CP1c possessed a dominant α-helical structure on both Mal SAM and Mal-OH SAM surfaces, as indicated by a deep minimum at ~207 nm and a shallower minimum at ~ 222 nm. The slight spectral difference between peptides immobilized on different surfaces may be caused by the slight difference of the amounts of peptides immobilized on the two surfaces or a slight difference of the ratio between random coil and helix. Spectra fitting and data analysis were performed using DICHROWEB server (http://dichroweb.cryst.bbk.ac.uk/), an online tool that contains spectra references of soluble proteins and membrane proteins. Such data analysis may not be very accurate because the method relies on empirical algorithms by comparing the CD spectra of peptide/protein being analyzed to the CD spectra of peptide/protein with known structures. Nevertheless, we believe that it is reliable to compare the structures of the same peptide/protein immobilized on different surfaces. The fitted helical components on the Mal SAM and Mal-OH mixed SAM surfaces are similar, both at ~52%.

Table 1. Amino acid sequence of CP1c

ID	Sequence
CP1c	H_2N-SWLSKTAKKLENSAKKRISEGIAIAIQGGPRC-COOH

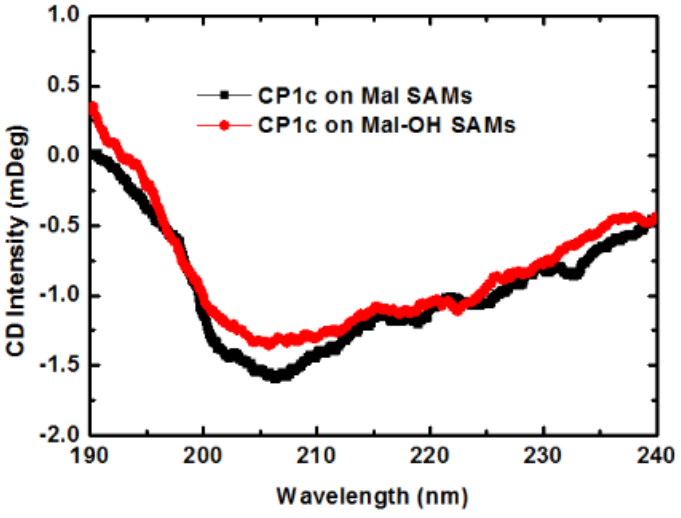

Figure 3. CD spectra of the surface immobilized CP1c on Mal SAM surface and mixed Mal-OH SAM surface in PBS.

2.2.2. Immobilization of Azide-mutated MI-78 Peptide.

The amino acid sequences of the wild type MSI-78 and terminal azide modified MSI-78 peptides are shown in Table 2. Studies showed that wild type MSI-78 adopts a random coil conformation in aqueous buffer solution (Fig. 4). Similar to the wild type MSI-78, the azido modified MSI-78 adopts a random coil conformation in aqueous buffer solution (not shown). After surface coupling through the reaction of the MSI-78 azido group with the surface alkyne group, the immobilized MSI-78 exhibited an alpha-helical conformation, as characterized by the double minima at 207 nm and 222 nm in the CD spectra (Fig. 4). The helix content of the surface bound nMSI-78 and MSI-78n are similar at ~84.5% and ~83.7%, as calculated by CDSSTR, SELCON3, and CONTIN (http://dichroweb.cryst.bbk.ac.uk/).

Table 2. Amino acid sequence of wild type MSI-78, nMSI-78 and MSI-78n

ID	Sequence
MSI-78	GIGKFLKKAKKFGKAFVKILKK-NH_2
nMSI-78	($-N_3$)KGIGKFLKKAKKFGKAFVKILKK-NH_2
MSI-78n	GIGKFLKKAKKFGKAFVKILKKK($-N_3$)-NH_2

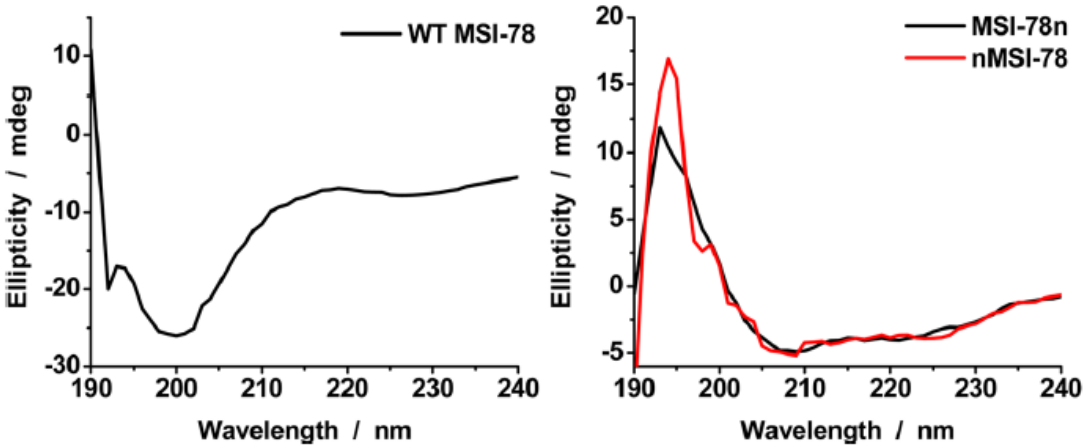

Figure 4. CD spectra of (left) WT MSI-78 in phosphate buffer; (right) surface immobilized nMSI-78m and MSI-78mn in aqueous phosphate buffer.

2.3. Environmental Effect on Surface Immobilized Peptide/Protein

2.3.1. Air Effect on Surface Immobilized Peptide/Protein

Cecropin A (1-8)-melittin (1-18) hybrid peptide (hybrid peptide, sequence can be found in Table 3) is immobilized on CVD polymer and maleimide SAM surfaces. Figure 5a is the collected CD spectra of hybrid peptide immobilized on CVD polymer in phosphate buffer and in air. Surface immobilized peptide showed similar secondary structure in solution and in air with two negative bands at 222 nm and 208 nm and a positive peak at 190 nm, which indicate that hybrid peptides immobilized on CVD polymer have α-helical structure. Figure 5b shows the CD spectra of hybrid peptide immobilized on maleimide SAM surface. The secondary structure in air formed more helical structure than in solution, suggested a structural transition from solution to air.

Table 3. Amino acid sequence of hybrid peptide.

ID	Sequence
Hybrid Peptide	H_2N- KWKLFKKIGIGAVLKVLTTGLPALISC -COOH

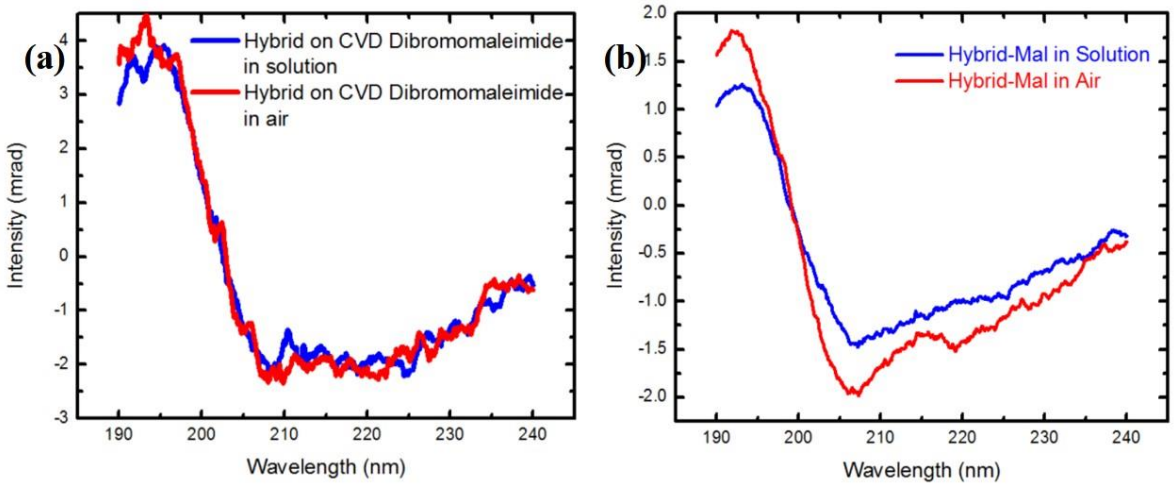

Figure 5. CD spectra of a) hybrid peptide immobilized on CVD polymer, and b) hybrid peptide immobilized on SAM surface.

2.3.2 Humidity Effect on Physically Adsorbed Peptide/Protein

CP1c and cysteine mutated Nitroreductase (NsfB-H360C) were physically adsorbed on OTS SAM surface and characterized secondary structure using CD with different relative humidity (RH) levels. Multiple layers of peptide and protein molecules were adsorbed on surfaces. The results in Figure 6 show that the secondary structures of both physically adsorbed CP1c and NsfB-H360C did not change under different humidity levels. We believe that here the inter-peptide/protein interaction plays an important role because multilayered peptide/protein samples were studied.

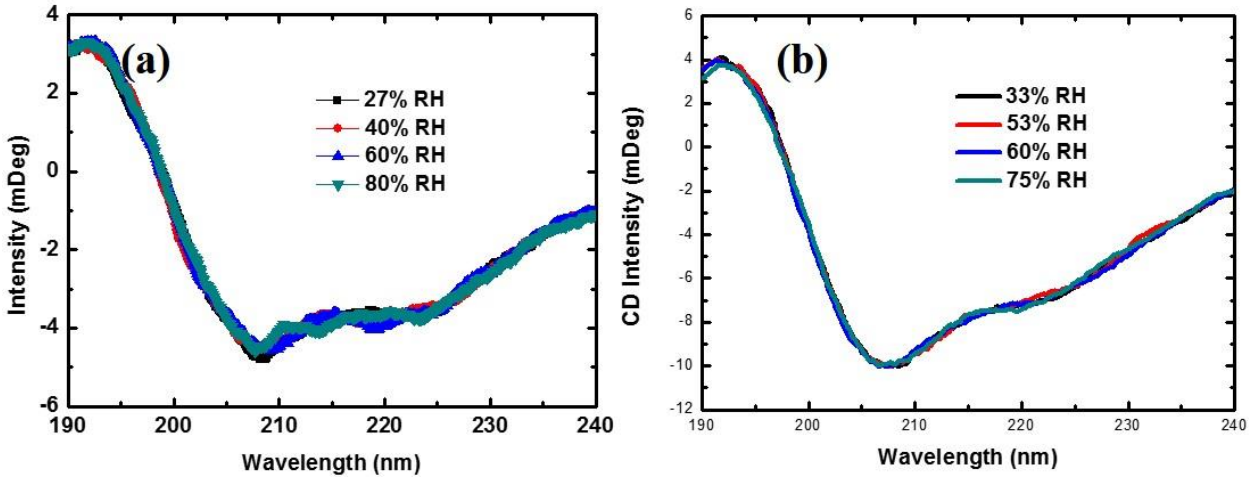

Figure 6. CD spectra physically adsorbed a) CP1c and b) NsfB-H360C at different humidity levels

2.3.3 Humidity Effect on Chemically Immobilized Peptide/Protein

CP1c and NsfB-H360C were chemically immobilized on maleimide SAM surfaces and CD spectra were collected from these surface immobilized molecules to study their secondary structures under different humidity levels. CP1c and NsfB-H360C formed a monolayer each on a SAM surface. Figure 7 indicates that the chemically tethered peptide/protein shows different secondary structures in solution and in air. By increasing the humidity, the secondary structures of immobilized peptide and protein become more similar to those in solution. The results suggested that environmental humidity influences the chemically tethered peptide/protein secondary structure.

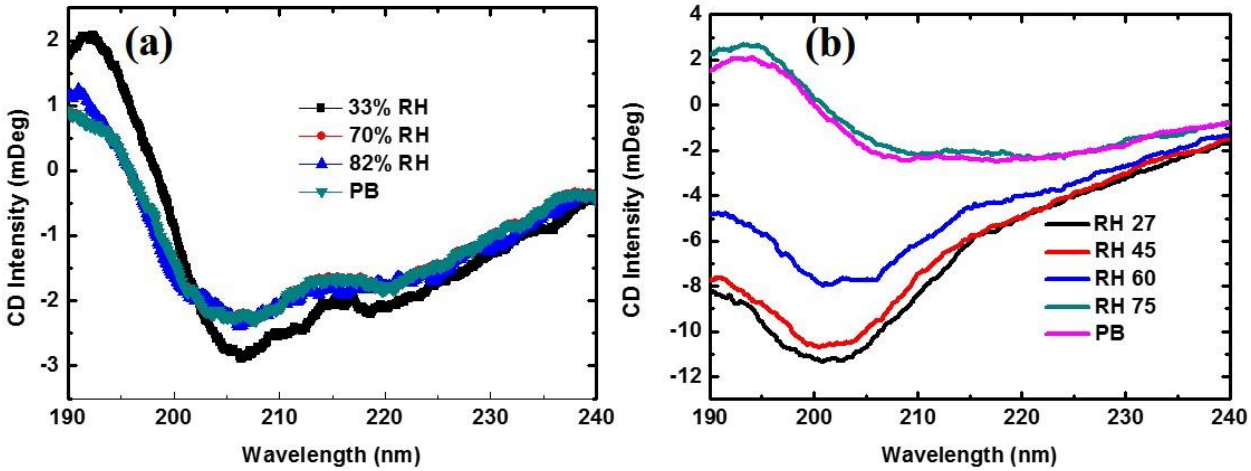

Figure 7. CD spectra of chemically immobilized a) CP1c under humid air and in solution and b) NsfB-H360C under humid air and in PB solution.

2.3.4 Secondary Structure of Surface Immobilized MSI-78 with Spin Coated Sucrose

When MSI-78 was immobilized on a SAM surface, its secondary structure can be retained by spin coated sucrose, as shown in Figure 8. Three different methods were used to coat sugar onto surface immobilized MSI-78. They all protected the secondary structure of immobilized peptides in air.

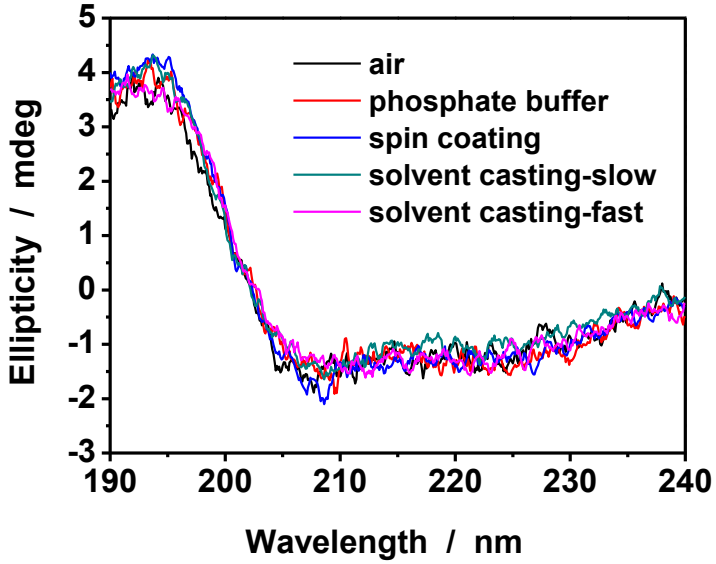

Figure 8. CD spectra of surface immobilized nMSI-78 in air, in phosphate buffer, with sucrose layer on top prepared using spin coating, solvent casting with the slow drying method, and solvent casting with the fast drying method.

2.3.5. Secondary Structure of Surface Immobilized NsfB with Polymers.

NsfB with two cysteines at positions 360 and 133 (NsfB-H360C-H133C double mutant) was immobilized by itself and co-immobilized with poly-sorbitol methacrylate on maleimide SAM surfaces. The purpose of this work is to see whether the hydromimetic poly-sorbitol methacrylate can protect protein secondary structure when the co-immobilized protein-polymer substrate is exposed to air. Results in Figure 9a show that the chemically immobilized NsfB-H360C-H133C double mutant on maleimide SAM by itself partially lost its secondary structure after the sample was exposed to air for 1 day. The co-immobilized NsfB-H360C-H133C double mutant and poly-sorbitol methacrylate surface shows identical secondary structure of NsfB before and after exposed to air 1 day (Fig. 9b), suggesting that the hydromimetic polymer preserved the secondary structure of surface immobilized protein after it was exposed to air for 1 day.

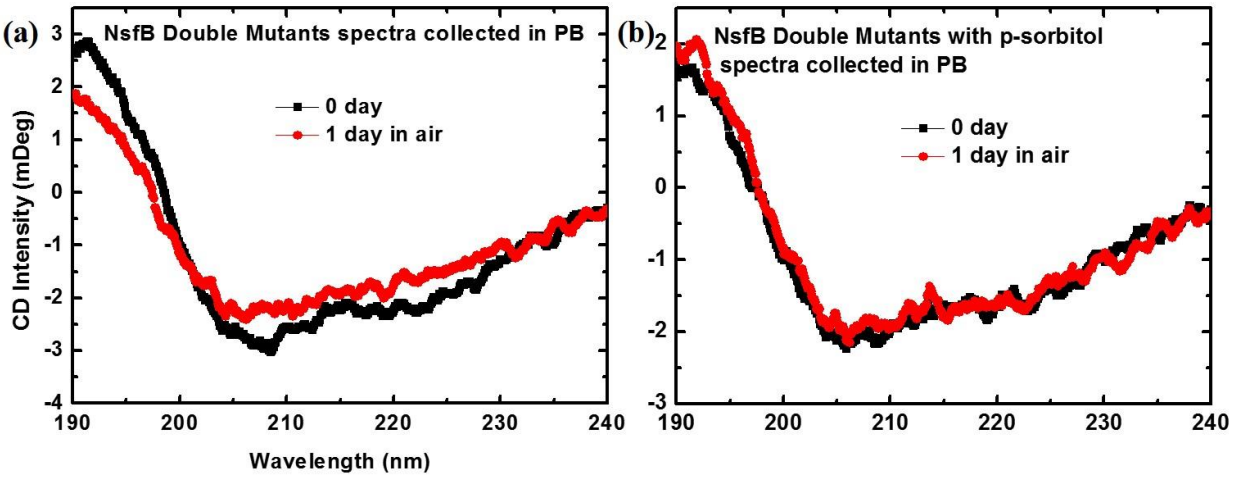

Figure 9. CD spectra of (a) chemically immobilized NsfB-H360C-H133C double mutant on maleimide SAM in PB solution before and after exposed to air for 1 day, and (b) co-immobilized NsfB-H360C-H133C double mutant with poly-sorbitol methacrylate on maleimide SAM in PB solution before and after exposed to air for 1 day.

3. Summary

In this research, we successfully set up a new CD spectrometer. Compared with other CD systems, this CD system built in the PI's lab has unique configurations for the detection of peptide/protein structures. The CD system can be used to measure the secondary structures of surface immobilized peptides and proteins, with outstanding signal-noise ratio for the detected CD signal. The custom-built air-tight sample compartment and humidity control-system provide well controlled humid environment for surface immobilized samples with an adjustable humidity level ranging from 25% to 99% (relative humidity). We successfully applied this robust and sensitive CD system to study surface immobilized peptides and proteins. We successfully studied secondary structures of surface immobilized peptides/proteins with different surface

immobilization methods, on different immobilization substrates, at different environmental conditions, and with or without co-immobilized hydromimetic functionalities. As we mentioned in the proposal, the elucidation of structure-function relationship of immobilized peptides and proteins provides important knowledge to aid in the design and development of materials and devices incorporating immobilized peptides and enzymes. Such materials and devices have many military and defense related applications. This method will be extremely useful to study structures of surface immobilized peptide/protein, providing important supplemental structure information that SFG and ATR-FTIR cannot provide.